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Methylation of Arsenic by Anaerobic Microbial Consortia Isolated from Lake Sediment

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Anaerobic enrichment cultures, isolated from arsenic-contaminated lake sediment in the Canadian sub-arctic and grown in five selective media, methylated arsenate/arsenite to produce mono-, di- and tri-methyl arsenicals. The extent of methylation and methylarsenic species produced varied with the type of enrichment. Iron-reducing, manganese-reducing, sulfate-reducing and broad-spectrum anaerobic heterotrophic mixed cultures all produced methylarsenicals. Sulfate-reducing cultures produced higher concentrations of methylarsenicals (especially trimethyl species) than iron- or manganese-reducers. There is evidence that several of the methylarsenicals, which were hydride-reactive at pH 6, were methylarsenic(III) thiols. The organoarsenicals produced by enrichment cultures were the same as those detected in the porewater of the lake sediments used to initiate the enrichment cultures. Overall, this study demonstrates that microbes from anaerobic lake sediments can methylate (and demethylate) arsenic, a capability shared by manganese-, iron-, and sulfate-reducing microbial consortia.

Keywords: Anaerobe, arsenic, microbial, organoarsenicals, sediment

INTRODUCTION

Methylated arsenicals are found in soil and in marine and lacustrine waters and sediment porewaters (for a review, see Ref. 1). Synthesis of these organoarsenicals via biomethylation has been primarily attributed to phytoplankton and algae, and their death and deposition purportedly accounts for organoarsenicals in sediments. However, a capacity for bacterial methylation in marine[2] and lacustrine sediments[3] has been demonstrated. The importance of this activity to the environmental arsenic cycle and to overall aquatic toxicity has not yet been established. Microbial methylation/demethylation, however, is an important influence on the bioavailability and toxicity of several other metals/metalloids, including mercury, lead and selenium.[4] Organoarsenicals constitute a major portion of the hydride-reactive arsenic found in some freshwater systems; Anderson and Bruland[5] documented methylated arsenic concentrations which comprised 1–59% of the total arsenic budget in water from California lakes or estuaries.

Monomethylarsinic acid (MMAA), dimethylarsonic acid (DMAA), trimethylarsine oxide (TMAO) and volatile arsines have been produced from arsenate and arsenite by aerobic laboratory cultures inoculated with fresh lake sediment and grown in a rich nutrient broth,[3] as well as by aerobic and anaerobic cultures inoculated with marine sediment and grown in marine broth.[2] These cultures were probably dominated by rapidly growing oligotrophs as there was no provision of special nutritional needs for more fastidious anaerobes, and in one study[3] oxygen was not excluded. Specific groups of bacteria in the suboxic zones of sediments (e.g. nitrate dissimilators, iron-, manganese- and sulfate-reducers, methanogens), however, may exhibit different potentials for biomethylation. For example, mercury methylation in soil and aquatic sediments is attributed primarily to sulfate-reducers.[6,7] In addition, the oxidation–reduction (redox) conditions of sediments exert control over the chemical form of inorganic arsenic[1] and distribution between particles and porewater. Within oxic and slightly suboxic zones, arsenic (as arsenate) is removed from solution via complexation with iron- and manganese-oxyhydroxides. In increasingly reduced soils or sediments, however, dissolved arsenic is released to the porewater (as

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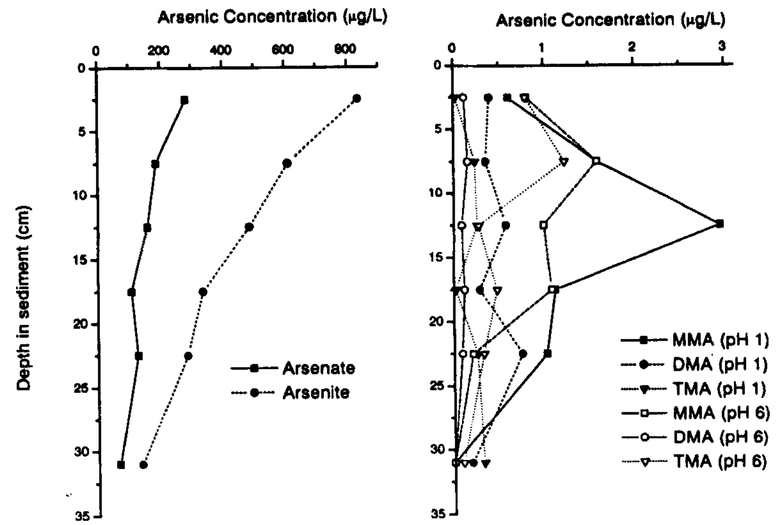


Figure 1 Vertical distribution of inorganic and methylated arsenicals in Kam Lake sediment porewater. MMA (pH 1) indicates monomethylarsenic acid after hydride generation at pH 1; DMA (pH 1) and TMA (pH 1) are dimethylarsonic acid and trimethylarsine oxide respectively, also hydride-reactive at pH 1. pH 6 species have the same retention times as their pH 1 counterparts but were hydride-reactive at near-neutral pH (see Analytical methods).

arsenite) and thus may be more bioavailable. Anaerobic microbes, therefore, may theoretically have greater requirement for detoxifying arsenic by methylating it.[1]

We have previously examined arsenic speciation in the water and sediment of Kam Lake (Northwest Territories, Canada), a lacustrine system with elevated concentrations of total arsenic[8] (i.e. up to 1630 $\mu g\, g^{-1}$ in surface sediment; 1120 $\mu g\, dm^{-3}$ in porewater; and 530 $\mu g\, dm^{-3}$ in the water column during August 1991). Natural concentrations of arsenic in the water column of lakes do not usually exceed 1–2 $\mu g\, dm^{-3}$.[1] Kam lake is adjacent to a gold mine effluent discharge and, in the 1970s, received an accidental spill of arsenic-rich mine tailings that has since been covered by natural sediment. MMAA, DMAA, TMAO and other hydride-reactive organoarsenicals were present in Kam Lake sediment porewater and surface water collected in August 1990 (Fig. 1). Although only a small portion of the total arsenic in surface sediment porewater was present as methylarsenicals (<2% of total arsenic), certain species occurred at elevated concentrations between the tailings layer and the surface. In particular, a maximum in the concentration of monomethylarsinic acid as measured after hydride generation at pH 1 (see Analytical methods) lay immediately above the tailings layer. This study examines the potential of bacteria of various nutritional groups isolated from Kam Lake sediments to methylate inorganic arsenicals.

EXPERIMENTAL

Microbes were isolated from arsenic-contaminated lake sediment into sterile broth media enriched with sodium arsenate. Following at least three enrichment cycles, a subset of the isolates was used to investigate arsenic methylating activity of different nutritional groups.

Isolation of arsenic-tolerant microbes from Kam Lake sediment

Sterile broth cultures (10 dm^3) enriched with sodium arsenate (100 $\mu g\, As\, dm^{-3}$) were inoculated with approximately 1 g of sediment taken at depths of 1, 5, 9, 13, 19 and 25 cm from a core collected from Kam Lake, N.W.T., Canada, in August 1991. The mine tailings formed a layer at 9–17 cm. Redox potentials were measured by using a platinum electrode inserted into the core immediately after collection: between 3 cm and 7 cm depth, the measured E_h decreased from −19 mV to −219 mV. The sediment core temperature, measured with a portable thermistor, decreased from 20.1 °C at the surface to 19.7 °C at 35 cm.

Seven nutritional types of bacteria were selected from each depth in the Kam Lake core. Media were prepared that were designed to be selective for (1) aerobic and anaerobic oligotrophs (Tryptic Soy Broth (TSB), 1/4 strength, Difco); (2) nitrate-dissimilators;[9] (3) iron-reducers;[10] (4) manganese-reducers;[10] (5, 6) sulfate-reducers (with acetate and lactate as alternative carbon sources);[11] and (7) potential arsenate-reducers (based on the medium of Lovely and Phillps[10]). All procedures were performed under anaerobic conditions: the GasPak system ($H_2 + CO_2$ generator envelopes: BBL Microbiology Systems) was used to produce the anaerobic incubation conditions in the original enrichment cultures. Pre-reduction of the sterilized media involved deoxygenation of water, addition of the appropriate mineral salts, autoclaving and addition of the filter-sterilized reducing agents sodium sulfide ($360\,\mathrm{mg\,dm^{-3}}$) as well as sodium dithionate ($30\,\mathrm{mg\,dm^{-3}}$). All procedures were carried out under inert-atmosphere conditions. Resazurin was added as a colorimetric redox indicator; it turns from blue to pink when E'^0 falls to -51 mV, and becomes colourless at E'^0 values of less than -100 mV.

All sediment core samples gave rise to microbial growth in all media except for the 1, 9, 19 and 25 cm depth samples inoculated into nitrate dissimilation broth. Enrichment cultures isolated from 5 cm sediment samples were subsequently chosen for methylation studies because aerobic oligotrophic cultures from this depth produced the highest levels of MMAA and DMAA from arsenate in lab cultivations.[12] The 5 cm samples lie immediately above the mine tailings layer where the microbial community may be adapted to high arsenic levels.

Investigation of methylarsenic species production under selective culture conditions

The net production of methylarsenicals (combined effects of methylation and demethylation) by bacterial consortia was examined under five separate selective culture regimes; isolates that were chosen for further investigation included anaerobic oligotrophic (TSB), iron-reducing, manganese-reducing and both sulfate-reducing consortia. Broth cultures of the same composition as those used for isolation of microbes, but spiked with $1000\,\mu\mathrm{g\,As\,dm^{-3}}$ rather than $100\,\mu\mathrm{g\,As\,dm^{-3}}$ of sodium arsenate, were prepared, pre-reduced, sterilized, and then inoculated using a small volume ($40\,\mathrm{cm^3}$) of the corresponding isolation/enrichment cultures. Methylation studies were conducted at room temperature in gas-tight $400\,\mathrm{cm^3}$ Teflon chambers ($300\,\mathrm{cm^3}$ culture media + $100\,\mathrm{cm^3}$ headspace) fitted with ports for removal of liquid and gaseous samples. The arsenic concentration used was extremely high relative to unperturbed (natural) lake sediment porewater concentrations, which are generally less than 100–200 $\mu\mathrm{g\,As\,dm^{-3}}$; however, it was within the range observed within Kam Lake sediment porewater (Fig. 1). Inocula were prepared from cultures that had passed through at least three enrichment cycles and were 72 h old upon addition. Uninoculated arsenate-amended broths were used as controls. Arsenite was detected in all controls: preparation of PRAS (pre-reduced anaerobically sterilized) media results in the reduction of some arsenate to arsenite, as would be expected under the redox conditions established.[1] The colorimetric redox indicator, resazurin, indicated that E'^0 was less than -110 mV in all culture chambers throughout the duration of the experiment. Samples of media were taken on days 4, 11, 14, 21, 27 and 35, using syringes fitted with 0.22 μm Teflon filters to exclude microbes and particulates.

Analytical methods

Arsenicals were analyzed using pH-selective hydride-generation/gas chromatographic/atomic adsorption spectroscopy techniques (HG GC AA) as reported previously.[2,8] Briefly, volatile arsines were measured on days 14 and 27 by completely flushing the vessel headspace with helium, cryo-trapping for 5 min in liquid nitrogen, separating individual arsines on a previously silanized Poropak PS 80–100 packed column (50 cm × 1 mm i.d.) in a programmable GC oven, and subsequent measurements by AA. The packed column was heated from 50 to 150 °C at a rate of $25\,°\mathrm{C\,min^{-1}}$. Aqueous arsenicals were similarly measured as volatile arsines after reaction of $10\,\mathrm{cm^3}$ aliquots of filtered (0.22 μm) culture media (which were diluted to $60\,\mathrm{cm^3}$ in a reaction vessel) with $5\,\mathrm{cm^3}$ 4% sodium borohydride. Borohydride reductions were carried out at two pH values: pH 6 which is selective for arsenite, followed by pH 1 for reduction of arsenic(V) and methylarsenic(V) species. The pH 6 reaction was run after addition of $2\,\mathrm{cm^3}$ of HCl–Tris buffer. After liberation of hydride-reactive arsenicals at pH 6, the reaction mixture was acidified to pH 1 with 3 cm^3 4 M HCl, and sodium borohydride was re-injected. The detection limits for all arsenicals ranged from 0.020 to 0.040 $\mu g\ dm^{-3}$. The analytical data for replicate samples exhibited less than a 20% relative standard deviation.

Arsenic metabolites are reported here as arsine derivatives produced subsequently to hydride-generation. Under this convention, MMA (pH 1) is monomethylarsine, often attributed to the reaction of monomethylarsenic acid (MMAA) with borohydride at low pH (pH 1 in this study).[4] Similarly, DMA (pH 1) and TMA (pH 1) are generally attributed to hydride reaction with DMAA and TMAO respectively.[4] This convention was chosen because significant amounts of methylated arsines were also measured after hydride generation at pH 6, and the specific identify of the precursor compounds has not been definitively established (see Discussion).

RESULTS

All enrichment cultures produced methylarsenicals, but the extent of methylation and the proportion of different species varied for different culture conditions. This is illustrated in Fig. 2 for days 4 and 35. The methylarsenical results in Figs 2 and 3 are presented as percentages of the total hydride-reactive dissolved arsenic (methyl species + arsenate + arsenite). Total hydride-reactive arsenic concentrations declined only slightly over the duration of the experiment: the total arsenic concentration in all culture media and controls ranged from 980 to 330 μg As dm^{-3} at the end of the experiment (day 35), whereas the original spiked concentration was 1000 μg As dm^{-3}. The decline in total arsenic concentration was most accentuated in iron- and manganese-reducing cultures (both in inoculated and control media), and in the inoculated TSB media. The slight decrease in total, hydride-reactive arsenic levels in the cultures, therefore, is attributable to physical adsorption–desorption phenomena rather than metabolism, since it was also observed in the controls. No hydride-reactive organoarsenicals were present in quantifiable concentrations ($<3\sigma$ of the baseline) in controls except in the TSB control, which was contaminated during the helium purge for the analysis of volatile arsines; the trace levels noted in some controls were near or below the analytical detection limit of approximately 0.020 $\mu g\ dm^{-3}$. The anaerobic oligotrophs (TSB cultures), the most active methylators, produced a high proportion of DMA (pH 1), i.e. DMAA prior to hydride generation.

The sulfate-reducing enrichments produced the second highest concentrations of methylarsenicals. The acetate enrichment initially produced mostly trimethyl species (TMA, pH 1; TMA, pH 6); however, the relative concentrations of mono- and di-methylarsenicals increased with time (Fig. 3). Lactate cultures produced only TMA (pH 6) by day 4, but a range of metabolites dominated by TMA (pH 1) resulted from extended incubation. These results demonstrate the time-dependent nature of the metabolism.

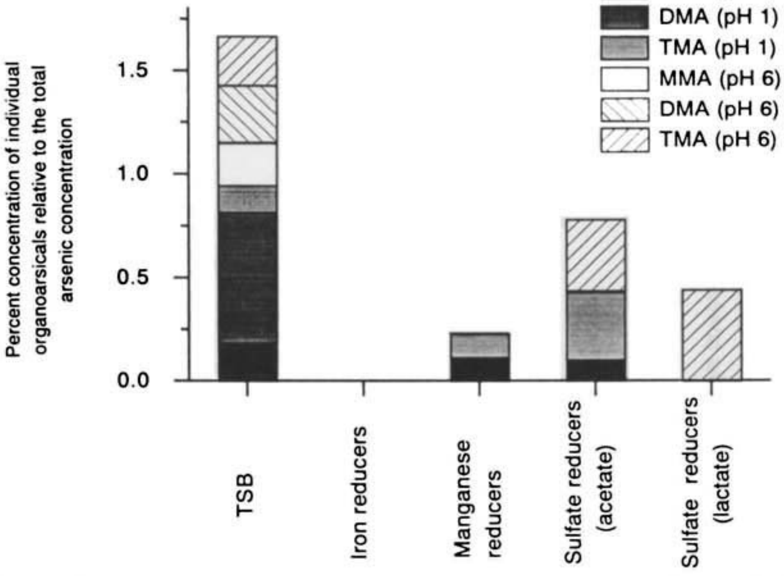
The iron- and manganese-reducing cultures grew poorly, a fact that may account for the low yields of methylarsenicals (Fig. 2). The production of arsenic metabolites, however, was not increasing at day 35, and some methylarsenic concentrations had peaked and declined. For example, Mn-reducers produced ~7 ppb (parts per 10^9) of methylarsenicals by day 11 but the concentration dropped to less than 2.5 ppb by day 27.

The production of volatile arsines was assessed qualitatively in the headspace of culture vessels on days 14 and 27. Several arsines were produced by cultures by day 14: AsH_3 and MMA (major volatile arsine) in sulfate-reducing (acetate) cultures; AsH_3 and TMA (major volatile arsine) in sulfate-reducing (lactate) cultures; and AsH_3, MMA and TMA in the TSB (contaminated) control. After 27 culture days, only the TSB test and control cultures contained detectable volatile arsines, and the only detectable product was TMA.

DISCUSSION

These experiments demonstrate that anaerobic isolated from lake sediment are capable of producing the organoarsenicals found in that sediment. Sulfate-reducing bacteria, previously identified as the principal methylators of mercury in lake sediment,[6,7] are also capable of producing organoarsenicals, and sulfate-reducing mixed bacterial enrichment cultures produced higher methylarsenic concentrations than iron- or manganese-reducing cultures. The role of iron- and manganese-reducers in environmental cycling

(a) 4 days incubation



(b) 35 days incubation

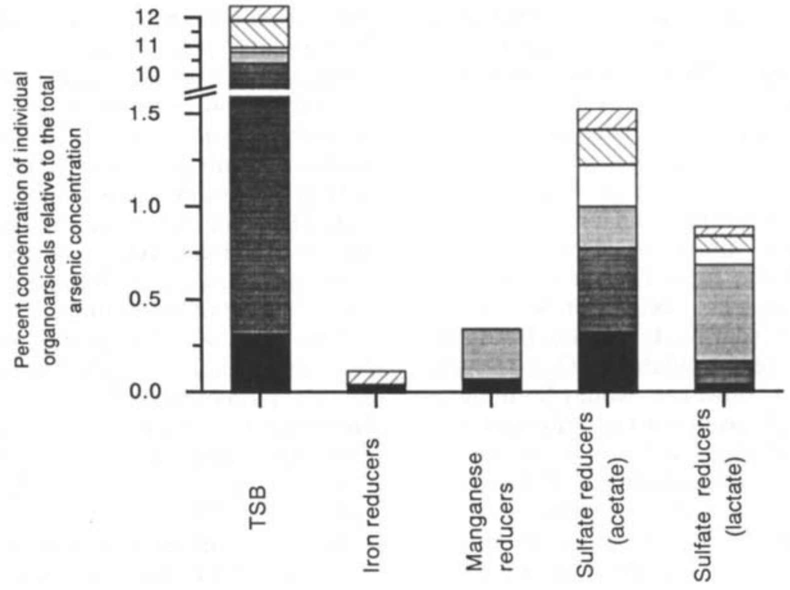


Figure 2 Methylarsenicals produced by enrichment cultures: samples taken after four days (a) and 35 days (b) of incubation.

of organoarsenicals cannot be discounted on the basis of these laboratory investigations, because all cultures were observed to produce methylarsenic species from arsenate/arsenite. In addition, these microbes contribute to the reductive dissolution of iron and manganese oxides, which results in desorption of arsenic (as arsenite) and increased arsenic bioavailability.[5, 11, 13] It was not possible to make a direct comparison of rates of arsenic methylation between different nutritional groups, since no data were collected on between-culture variations in growth or metabolic rate.

The enrichment cultures simultaneously contained several methylarsenicals, and some also produced volatile arsines [TSB and sulfate-reducing (lactate) cultures]. The TSB culture, a broad-spectrum, rapidly growing collection of facultative and/or obligate anaerobes, contained

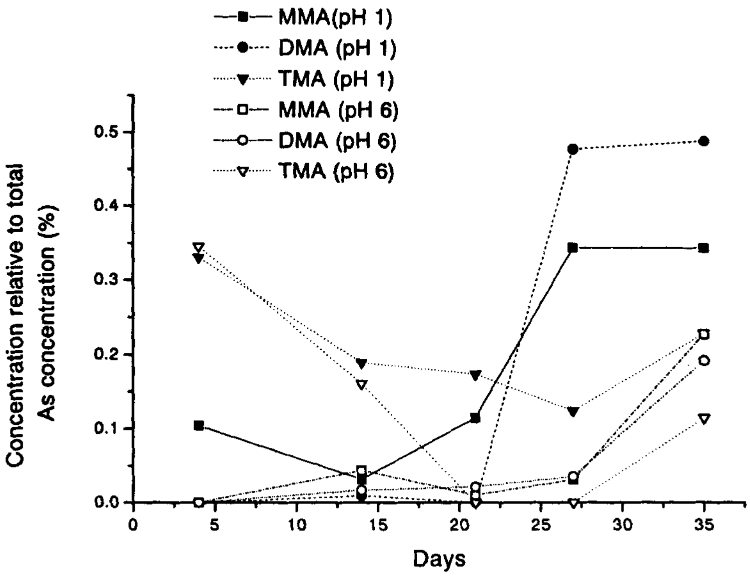


Figure 3 Methylarsenical production by sulfate-reducing (acetate) enrichment cultures.

up to 12.3% of the aqueous arsenic budget as methylarsenicals (Fig. 2). The dominant methylarsenical by far was DMA (pH 1), which may relate to the presence of DMAA as a major organoarsenical in Kam Lake cores and its dominance in other lacustrine samples.[1,5]

Methanogens have also been implicated in methylarsenical formation in anaerobic environments, and selective inhibition of methanogenic activity decreased the formation of arsine from arsenate.[14] Methanogens were not specifically examined in the present study, although suitable growth substrates were available ($CO_2 + H_2$, acetate). Their impact, however, would be minimal since they are not competitively dominant in sulfate-reducing cultures, and are normally cultured using higher concentrations of hydrogen and carbon dioxide. A study using specific inhibitors, such as that conducted by Kerry *et al.*,[7] would help to delineate the spectrum of active methylators.

The change in relative concentrations of methylarsenic compounds in sulfate-reducing cultures over the cultivation period followed a production/decay pattern (e.g. Fig. 3) that may be attributable to sequential methylation and demethylation, as has been suggested in other studies.[2,3] This could have resulted from a succession of different species in these cultures, and/or from shifts in the biochemical activities of specific bacterial strains. Additional experiments with pure microbial cultures will help to resolve this. In particular, it is not clear whether mono- and dimethylarsenicals are produced *de novo* or by demethylation of trimethylated species released as exometabolites. The sulfate-reducing (acetate) culture initially produced TMA (pH 1) and TMA (pH 6) as the dominant methyl species (day 4, Fig. 3), which subsequently declined in concentration. Monomethylarsenic acid and dimethylarsonic acid were the dominant methylarsenicals after 21 days of incubation.

This study was designed to assess the potential for, rather than the quantitative importance of, arsenic biomethylation in lacustrine sediments. However, the results bear a similarity to sediment porewater data from a Kam Lake core which revealed the presence of MMA (pH 1), DMA (pH 1) and TMA (pH 1), as well as similar peaks from parent organoarsenicals which are hydride-reactive at pH 6 (Fig. 1). The percentage concentrations of methylarsenicals in sulfate-reducing cultures (e.g. day 35 data) relative to the total arsenic (organic plus inorganic) concentrations were also similar to those found in the sediment from which the bacteria were isolated (i.e. the sum of methylarsenicals ranged from 0.1 to 1% of total arsenic). Given the high levels of anthropogenic arsenic, it is possible that microbes indigenous to, and isolated form, Kam Lake may have adapted in a manner to minimize arsenic stress, via an enhanced ability to methylate arsenic. It would be interesting to know whether bacteria in anaerobic sediments which are not contaminated by arsenic share similar abilities.

In these experiments, significant amounts of methylated arsines were produced by hydride generation at pH 6. The same is also true for samples taken from Kam Lake cores. Borohydride treatment reduces arsenite to arsine (AsH_3) at pH 6; arsenate and some methylated arsenicals [$(CH_3)_nAsO(OH)_{3-n}$] are reduced to more volatile AsH_3 and $(CH_3)_nAsH_{3-n}$ respectively at pH 1 ($n = 0$–2). Pure standards of MMAA or DMAA made up in sterilized, de-ionized water do not produce volatile arsines when reacted with borohydride at near-neutral pH. However, the methylarsenic thiols dimethylarsinoglutathione and monomethylarsinocysteine (synthesized and supplied by Cullen *et al.*[15]) reacted during HC GC AA only at pH 6 to produce arsenic peaks identical in retention time to DMA and MMA respectively. The synthesized arsinothiols are unstable to oxygen.[15] This emphasizes the necessity for oxygen-free techniques in the preservation of environmental samples if arsinothiols are to be detected, and may help to explain why these compounds have not been previously detected in exocellular environmental samples.

Reimer[2] and others proposed that methylarsenic(III) thiols of the form $(CH_3)_nAS^{III}(SR)_{3-n}$ (n is 1–3 and R is H or an organic moiety) and $[(CH_3)_2As^{III}]_2S$ exist in exocellular environmental compartments such as sediment porewater. Others have demonstrated the presence and importance in sediments of thiols (including 3-mercaptopropionate, cysteine, glutathione and methanethiol), that move between a sediment-bound pool and the porewater, and are microbially metabolized in the aqueous phase.[16] We speculate, as have others,[1] that methylated arsinothiols may be formed under anaerobic conditions. The production of arsenothiols could be mediated biologically, or strictly chemically following the production of methylarsenic(V) compounds such as DMAA or TMAO. For example, DMAA reacts with hydrogen sulfide, a by-product of sulfate-reduction, to form $[(CH_3)_2As^{III}]_2S$ and highly insoluble sulfur-containing complexes.[15] Certainly, there is sufficient sulfur available in all of the culture media to account for the observed microgram-per-liter levels of pH 6 methylarsines. This paper provides, for the first time, circumstantial evidence of the existence of arsinothiols in the environment. Additional studies are in progress to identify these compounds by mass spectrometry.

Overall, our results demonstrate that arsenate is methylated by a variety of microbial consortia, that there are differences in the spectra of the methylarsenicals formed, and that the metabolites produced are found at similar concentrations in the porewater of Kam Lake sediment.

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